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Pentacyclic triterpenes and crude extracts with antimicrobial activity from Cameroonian brown propolis samples

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ABSTRACT

The aim of this study was to isolate compounds from Cameroonian propolis extracts and to test their activities against bacteria isolated from carcass at the Yaoundé slaughterhouse. The *n*-hexane, ethyl acetate and ethanol extracts of propolis samples from Ngaoundal and Tala-Mokolo were separated by successive silica gel column chromatography to give six triterpenes. Their structures were determined as 25-cyclopropyl-3β-hydroxyurs-12-ene (**7**), cycloart-3β-hydroxy-12, 25(26)-diene (**8**), lup-20(29)-en-3-one (**9**), olean-12-en-3β, 28-diol (**10**), lup-20(29)-en-3β-oate (**13**) and 3β-hydroxylup-20(29)-ene (**14**). Compounds 7 and **8** were new triterpene derivatives while 10 and **13** were isolated for the first time from propolis. The structures of all the compounds were established on the basis of spectroscopic analysis. Phytochemical screening of the methanol extract (**5**) revealed the presence of alkaloids, reducing compounds, coumarins, saponins and tannins accounting for its broad spectrum antibacterial activities. The six isolated compounds and crude extracts were tested for antimicrobial activity against some Gram negative bacteria. The methanol extract (**5**) of propolis samples was active against *Escherichia coli* and *Pseudomonas aeruginosa* (MIC: 0.2 mg/ml) whereas the isolated compounds **7**, **8** and **10** exclusively exhibited antimicrobial activity against *Salmonella*s pp (MIC: 0.1-0.15 mg/ml). The MIC values of all the four propolis products were greater than that of the standard drug (Amoxicillin): 0.1-0.2 mg/ml versus 0.4 mg/ml. Nevertheless, further pharmacological and toxicity studies on experimental animals are necessary to establish the safety of the propolis products for its use as topical antimicrobial agents.

INTRODUCTION

Propolis (bee glue) is a plant derived product. It is a resinous, strongly adhesive natural substance, collected by honeybees (*Apis mellifera* L.) from buds, leaves and stem barks of some trees and plants, mixed with pollen as well as enzymes secreted from the saliva of bees (Marcucci, 1995; Trusheva *et al.*, 2010). Bees use it as a general-purpose sealer, to smooth out the internal walls of the hive and as a protective barrier against intruders (Burdock, 1998). Propolis has been used as a remedy since ancient times. At present, propolis is a popular remedy in the folk medicine of several countries and a raw material for numerous preparations, health foods and beverages (Trusheva *et al.*, 2010). Propolis has been proved to possess valuable biological activities: antimicrobial, antiviral, anti-inflammatory, antioxidant, anaesthetic, immuno-stimulatory, anti-mutagenic and healing activities enabling regeneration of dead tissues (Banskota *et al.*, 2001 ; El-Kott and Owayss, 2008; Galvao *et al.*, 2007 ; Rebiai *et al.*, 2011; Sforcin, 2007 ; Trusheva *et al.*, 2010;). Due to its varied properties, propolis is known to be an extraordinarily efficient natural product for the treatment of common pathologies (Trusheva *et al.*, 2003; 2010; Popova *et al.*, 2009). It is known to enhance immune system probably due to its high antioxidant content (Kim *et al.*, 2008). However its biological properties depend on its chemical composition which in return is influenced by geographical and botanical factors (Ahn *et al.*, 2007; Popova *et al.*, 2005, 2009).

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Despite the chemical variability, it always demonstrates considerable biological activity, especially antimicrobial activity (Popova *et al*., 2005, 2009). More than 300 compounds, among which polyphenols, terpenoids, steroids, sugars, vitamins and amino acids have been detected in raw propolis (Trusheva *et al*., 2003 ; 2010 ; Popova *et al*., 2009; Kalogeropoulos *et al.*, 2009). For this reason, propolis from unexplored regions attracts the attention of scientists in the search for new bioactive molecules (Trusheva *et al*., 2010). In Cameroon, the production of propolis is still negligible while its consumption is on the increase. However little is known about the biological properties (antimicrobial and antioxidant activities) of Cameroonian propolis. So far, the available data on the characteristics of Cameroonian propolis were the work of Mbawala *et al.* (2010) and Seidel *et al.* (2008) on the antimicrobial activities of the ethanol extracts from two different regions. Moreover, there is only one report concerning compounds isolated from Cameroonian propolis (Talla *et al*., 2013). In this paper, we report the isolation, structure elucidation and antimicrobial activity of six crude extracts (**1-6**), two new cycloartane-type triterpenes (**7** and **8**) along with four known triterpenes (**9**, **10, 13** and **14**) from Cameroonian propolis.

MATERIALS AND METHODS

General experimental procedures

NMR spectra (^{1}H, ^{13}C, ^{1}H-^{1}H COSYqf45, HSQC, HMBC and DEPT135) were recorded on a Bruker AV500 spectrometer (500 MHz for ^{1}H and 125 MHz for ^{13}C) in $CDCl_3$ and TMS as internal standard. Chemical shifts were given in parts per million (ppm) and coupling constants in hertz. ESI-MS spectra (ionization voltage 3kV) were measured on a Q-TOF Ultima spectrometer (Waters). Column chromatography (CC) was performed on silica gel normal phase 60 (Merck, 63-200 µm) with step gradients of n-hexane-EtOAc and EtOAc-MeOH as eluents. Analytical TLC was performed on silica gel fixed on alumina plates (20 x 20 cm). Detection of the spots was achieved under UV light (254 and 366 nm) and by spraying with 50% sulfuric acid followed by heating at 100 °C.

Propolis samples

Propolis samples were collected from three localities belonging to two different agro-ecological zones of Cameroon: Meiganga and Ngaoundal (Adamawa region in 2008 and 2009 respectively) and Tala-Mokolo (Far-North region in 2009). Propolis samples were identified by Professor Fernand-Nestor T.Fohouo, Entomologist and beekeeper at the University of Ngaoundéré, Cameroon.

Extraction and isolation

Propolis from Ngaoundal was powdered (950 g) and extracted three times (6 L x 3) in methanol at room temperature for one week (48 h x 3). After each filtration, the solvent was evaporated under reduced pressure with a rotary evaporator (Büchi, 461). A brown crude methanol extract (575.2 g) was obtained and was successively partitioned with n-hexane and ethyl acetate to yield 255.1 and 125.6 g respectively. The remaining dry residue was 165.8 g. The extraction yield was calculated (Table 1). The other propolis samples were extracted following the same technique.

Then n-hexane dry extract (180 g) was subjected to silica gel column chromatography (Ø 0.063-0.200 mm, 700 g) and eluted with the mixtures n-hexane-EtOAc and EtOAc-MeOH in order of increasing polarity (0-100%) to yield a total of 216 fractions of 250 ml each. These fractions were combined on the basis of their TLC profile into seven major fractions (A-G). D (60-62) and E (67-75) which were major fractions were precipitated in the mixture n-hexane-EtOAc (9:1). Filtration and recrystallisation of these fractions yielded 350 and 200 mg of white powder of 25-cyclopropyl-3β-hydroxyurs-12-ene (**7**) and cycloart-3β-hydroxy-12, 25(26)-diene (**8**) respectively. The other fractions were subjected to silica gel column chromatographic purification using the mixtures n-hexane-EtOAc and EtOAc-MeOH with gradient polarity (0-100%) as eluent.

Ethyl acetate extract (100 g) was subjected to silica gel column chromatography (Ø 0.063-0.200 mm, 375 g) and treated following the same procedure as the n-hexane extract. Major fractions of A (6-9) gave 650 mg of white powder of lup-20(29)-3-one (**9**).

Moreover, methanol dry residue extract (150 g) was also subjected to silica gel column chromatography (Ø 0.063-0.200 mm, 650 g) and eluted with the mixtures n-hexane-CH_2Cl_2 and CH_2Cl_2-MeOH in order of increasing polarity (0-100%) to yield a total of 143 fractions of 250 ml each. These fractions were combined on the basis of TLC analysis into ten major fractions (A-J). Fraction A was subjected to silica gel column chromatographic purification (Ø 0.063-0.200 mm, 500 g) using the mixtures n-Hexane-EtOAc and EtOAc-MeOH with gradient polarity (0-100%) as eluent. Sub-fractions were combined on the basis of TLC analysis into sixteen serial profiles (A'-P'). A white powder was formed in F' (69-70) series from the mixture n-hexane-EtOAc (9:1). After filtration and recrystallization, 151 mg of olean-12-en-3β, 28-diol (**10**) was obtained.

Propolis collected from Meiganga was powdered (800 g) and extracted by maceration method under mechanical agitation. As soon as processed, powder (800 g) of propolis sample was transferred to 2.4 L for each solvent type in a glassware container. A rotating metallic rod was applied to the mixture for 4h to fasten the extraction process using electric current. The mixture was then allowed to settle for few minutes before it could be filtered under vacuum. The filtrate was concentrated using rotary evaporator. Later, the obtained dried extract was harvested and kept in a container for further analysis. The residue left on the separating funnel was removed and subjected to two further extractions with the same solvent yielding the final product. Three different solvents and one mixture were used for the extraction in order of increasing polarity: hexane, ethyl acetate, methanol and methanol/water (80/20, v/v). The extraction yield was then calculated (Table 2).

n-Hexane dry extract (150 g) was subjected to silica gel column chromatography (Ø 0.063-0.200 mm; 650 g) and eluted with the mixtures n-hexane-acetone and acetone-MeOH in order of increasing polarity (0-100 %) to yield a total of 91 of 300 mL each. These fractions were combined on the basis of TLC analysis into sixteen major fractions (A-P). Fraction C (8-16) was precipitated in the mixture of n-hexane-EtOAc (9:1). After recrystallization, 800 mg of white powder was obtained and its structure was not elucidated (**11**).

Fraction A (1-5) was subjected to silica gel column chromatographic purification (Ø50 – 200 mesh, Lab Tech Chemicals; 450 g) using the mixture n-hexane-EtOAc with gradient polarity (0-100 %) as eluent. Sub-fractions were combined on the basis of TLC analysis into fourteen major fractions (A'-N'). A white powder was formed in B' (4-8) series from n-hexane-EtOAc (9.5:0.5) mixture. After filtration and recrystallization, 1.9 g of pure compound was obtained and its structure was not determined (**12**). Propolis powder (68.4 g) from Tala-Mokolo was extracted three times (1L x 3) with ethanol at ambient temperature for one week (48h x 3). The solvent was removed under reduced pressure with a rotary evaporator and dry ethanol crude extract (53.8 g) was obtained. The extraction yield was calculated (Table 3).

Ethanol crude extract was further subjected to silica gel column chromatography (Ø 0.063-0.200 mm; 250 g) and 134 fractions of 150 ml each were collected and further treated following the same procedure as n-hexane extract of propolis sample from Ngaoundal. Fractions A(3–7) yielded 130 mg of white powder of lup-20(29)-en-3β-oate (**13**) and C(18 – 24) 280 mg of white powder of 3β-hydroxylup-20 (29)-ene (**14**). Moreover, the obtained crude extracts and isolated compounds (Table 4) were subjected to phytochemical screening (Table 5) and antimicrobial activities (Table 7).

Microbiological assay

Bacterial strains and culture preparations

In this study, *Salmonella* spp, *Escherichia coli* and *Pseudomonas aeruginosa* were isolated from carcass at the Yaoundé slaughterhouse in 2011 according to ISO 6579(ISO, 2002). The bacterial strains were maintained on Nutrient Agar slants at 4 °C in the Microbiology Unit of the Laboratory of Food Analysis and Quality Control, Institute of Medical Research and Medicinal Plants Studies (IMPM). Bacterial suspensions were prepared from loops primarily in buffered peptone water (Oxoid) by incubating at 37 °C overnight. Cultures were then transferred into Nutrient Agar plates (Difco) and incubated at 37°C overnight. For the assay, organisms were subcultured once onto fresh Nutrient Agar and inocula were prepared by transferring colonies to buffered peptone water (9 mL). Following incubation for 2–4 h at 37 °C, bacterial suspensions were adjusted to a turbidity equivalent to a McFarland 0.5 standard by adding sterile buffered peptone water. A sterile swab was immersed in the bacterial suspension and then pressed onto the wall of the tube to remove excess inocula. Finally the swab was streaked over the entire surface of the Nutrient Agar plates.

Agar plate diffusion assay using analytical paper discs

Preparation of stock solution for the test dilution

Each dried product (1 mg) was weighed and dissolved into 1mL of 10% DMSO following the method of Kar (2008) in a test tube. Six propolis samples crude extracts from Ngaoundal, Meiganga, Tala-Mokolo and six pure compounds isolated from n-hexane, ethyl acetate, ethanol and methanol extracts (propolis from Ngaoundal and Tala-Mokolo) were selected for the experiment.

Impregnation of filter-paper discs

Previously analytical paper discs (Ø.12.7mm, Schleider & Schuel, USA) were heat-sterilized at 160°C for 1 h in a hot oven. The discs were then soaked overnight in the stock solution. The solvent was later allowed to evaporate from the discs at 50 °C in a safety cabinet. For each experiment, control disc with pure solvent was used as blind control. The paper discs were applied to the agar plates using a sterilized forceps. To avoid overlapping of the zones of inhibition and possible error in measurement, discs were distributed 24 mm from each other and from the edge of the plate. After 24 h of incubation at 37°C, diameters of zones of growth inhibition were measured in millimetre as described by CLSI (2005). The experiments were carried out in duplicates.

RESULTS AND DISCUSSION

Extraction yields of propolis crude extracts

The overall extraction and isolation of propolis samples from Ngaoundal, Meiganga and Tala-Mokolo are shown in tables 1, 2, 3 and 4 below.

Table. 1: Extraction yield of crude extracts of propolis from Ngaoundal.

Extract code	Total weight of crude extract (g)	Extraction yield (%)
PMCE	575.2	60.5
PHEn	255.1	26.8
PEAEn	125.6	13.2
PMRE	165.8	17.4
Total extraction yield (%)		57.4
Lost Mass of crude extract after partition	28.7	3.1

PMCE: Propolis Methanol Crude Extract; PHEm: Propolis n-Hexane Extract; PEAEn: Propolis Ethyl Acetate Extract; PMRE: Propolis Methanol Residue Extract.

Table . 2: Extraction yield of crude extracts of propolis from Meiganga.

Extract code	Total weight of crude extract (g)	Extraction yield (%)
PHEm	358.4	44.8
PEAEm	189.5	23.7
PME	28.2	3.5
PMWE	10.1	1.3
Total extraction yield (%)		73.3

PHEm: Propolis n-Hexane Extract; PEAEm: Propolis Ethyl Acetate Extract; PME: Propolis Methanol Extract; PMWE: Propolis Methanol-Water Extract (80/20, v/v).

Table. 3: Extraction yield of crude extracts of propolis from Tala-Mokolo.

Extract code	Total weight of crude extract (g)	Extraction yield (%)
PECE	53.8	78.6

PECE: Propolis Ethanol Crude Extract.

Table. 4: Extracts and pure compounds obtained from propolis samples.

Propolis samples	Extract code.	Weight of extract (g).	Yield (%)	Isolated compounds
Ngaoundal	PHEn (**1**)	255	26.8	(**7**)
				(**8**)
	PEAEn (**2**)	125.6	13.2	SMZ6 (**9**)
	PMRE (**3**)	165.8	17.4	PS26 (**10**)
Meiganga	PHEm (**4**)	358.4	44.8	PHK1 (**11**)
				PHK4 (**12**)
	PME (**5**)	189.5	23.7	NF
Tala-Mokolo	PECE (**6**)	53.8	78.6	EN3 (**13**)
				EN18 (14)

NF: Non fractioned.

The total extraction yields were 57.4, 73.3 and 78.6% respectively. This could be due to the capacity of the different solvents to extract compounds with respect to their polarity. n-Hexane gave the highest yield of extraction (26.8%) (Table 1) and 44.8% (Table 2). This result is higher than that recorded in the literature (Alencar *et al.*, 2007; Oldini *et al.*, 2011). This could explain the richness of propolis samples from both collection sites in organic compounds that are weakly polar. Moreover, the percent yield of propolis methanolic extraction (3.5%) is lesser than that previously recorded (Bankosta *et al.*, 2001). However, the percent yield of ethyl acetate extraction (23.68%) is similar to previously reported (20.03 %) (Petrova *et al.*, 2010). On the basis of percent yield of propolis extraction, it clearly appears that the studied propolis contains more compounds of weak polarity than those recorded by other authors.

The propolis samples from Meiganga and Ngaoundal were subjected to two different extraction techniques: simple maceration at room temperature and maceration with mechanical agitation using a rotating rod. The latter gave greater yields for all solvents except the percent yield of methanol extract (3.5% and 17.4% for propolis samples from Meiganga and Ngaoundal respectively). The lowest yield of methanol extract (3.5%) recorded by the propolis samples from Meiganga may be attributed to the existence of weakly and moderately polar organic compounds in the methanol crude extract; that is, extraction with n-hexane and ethyl acetate had not been exhausted like in the case of maceration with mechanical agitation.

All the extracts and some isolated compounds were submitted to phytochemical screening and the results are reported in Table 5, reveals the presence of alkaloids in the hexane, ethyl acetate and methanol extracts for the propolis samples collected from Meiganga whereas only ethyl acetate extract was positive for its presence for propolis from Ngaoundal. Moreover, with the exception of the presence of anthraquinone in the hexane extract of propolis from Ngaoundal, phenolic compounds were present in the ethyl acetate, methanolic residual extracts and ethanolic crude extract from Ngaoundal and Tala-Mokolo propolis respectively. Coumarins were positive in hexane, ethyl acetate extracts from Ngaoundal and Meiganga propolis. They were also present in methanol, ethanol extracts of Ngaoundal and Tala-Mokolo propolis. Flavonoids were not detected from the propolis samples of the three collection sites (Meiganga, Ngaoundal and Tala-Mokolo). While reducing compounds were present only in propolis from Ngaoundal and Meiganga, saponins and volatile oils were detected in the three collection sites. Steroids were exclusively found in propolis samples collected from Meiganga and Tala-Mokolo. Propolis samples from the three collection sites were rich in triterpenes. While alkaloids were the most important compounds in propolis samples from Meiganga, triterpenes were the major chemical substances in propolis from the three collection sites. Generally, the propolis extracts in this study were poor in phenolic compounds and flavonoids. These findings differ from the results of other studies that showed phenolic compounds and flavonoids as the major components of propolis contributing to the biological activities (Burdock, 1998; De Castro, 2001, Marcucci *et al*., 2000; Tazawa *et al*., 1999; Uzel *et al*., 2005). On the other hand, the high terpenes content of Cameroonian propolis irrespective of the collection site indicates its similar chemical composition to the European and Mediterranean types (Popova *et al*., 2009, 2010b). The observed variation of phytochemical screening of propolis samples from the three collection sites confirms the fact that the chemical composition depends on geographical and botanical factors (Ahn *et al*., 2007; Popova *et al*., 2005, 2009).

Compounds isolated from propolis crude extracts collected from Ngaoundal and Tala-Mokolo

The structures of isolated compounds were elucidated on the basis of spectroscopic data (^{1}H, ^{13}C NMR and HSQC, HMBC, DEPT135, ^{1}H-^{1}H COSY). Compounds 25-cyclopropyl-3-β-hydroxyurs-12-ene (**7**) and cycloart-3-β-hydroxy-12,25(26)-diene (**8**) are new natural products. Comparison of the data with those reported in the literature led to the identification of the four other compounds as lup-20(29)-en-3-one (**9**) and 3β-hydroxylup-20(29)-eneor lupeol (**14**) (Talla *et al.*, 2013), olean-12-en-3β,28-diol also known as erythrodiol (Mahato and Kundu, 1994) (**10**) and lup-20(29)-en-3β-oate (Chatterjee *et al*., 2006) (**13**). Compounds **10** and **13** were isolated for the first time from propolis. The structures of the isolated compounds are shown in Fig.1.

Compound **7**showed a pseudo-molecular ion $[M+H]^+$ at *m/z* 425.50, $[M+Na]^+$ at *m/z* 447.47, and $[2M+Na]^+$ at *m/z* 871.98 in its ESI-TOFMS and the presence of 30 carbons in the ^{13}C NMR spectrum (Table 6), suggesting a molecular formula $C_{30}H_{48}O$. The ^{1}H-NMR spectrum displayed signals of a set of methylene protons at δ 0.18 and 0.39 (both d, J = 5.8 Hz), characteristic for cyclopropane ring, seven methyl groups at δ 0.65 (s), 0.66 (s), 0.66 (d), 0.75 (d), 0.98 (s), 1.05 (s) and 1.16 (s), one olefinic proton at δ 5.42 (dd, J = 3.6, 2.3 Hz) and one oxymethine at δ 3.12 (dd, J = 6.4, 9.2 Hz). The presence of 30 carbons (^{13}C NMR), from which

Table. 5: Phytochemical screening of propolis crude extracts and some isolated compounds.

Collection site/year	Extract code	Yield (%) & weight (mg)	Alkaloids	Anthraquinones	Phenolic compounds	Reducing compounds	Coumarins	Flavonoids	Saponins	Steroids	Triterpenes	Tannins	Volatile oils
Meiganga Propolis / 2008	PHE**4**	44.8	++	NT	Traces	-	+	-	-	+	++	-	+++
	(**11**)	800	-	NT	-	-	-	-	-	-	+++	-	-
	(**12**)	1900	-	NT	-	-	-	-	-	-	+++	-	-
	PEAE	23.7	++	NT	NT	+	+	-	-	+	++	-	-
	PME **5**	3.5	+	NT	NT	+	+	Traces	+	-	-	+	-
	PMWE (80:20)	1.3	-	NT	NT	-	-	Traces	+	-	-	++	-
Ngaoundal Propolis/ 2009	PHEn**1**	26.8	-	++	-	-	+	-	-	Traces	++	NT	++
	PEAE**2**	13.2	+	-	+	+	++	-	-	Traces	+	NT	-
	PMRE**3**	17.4	-	-	+	-	Traces	-	++	-	+	NT	-
	(7)	350	-	-	-	-	-	-	-	-	+++	NT	-
	(8)	200	-	-	-	-	-	-	-	-	+++	NT	-
	(9)	650	-	-	-	-	-	-	-	-	+++	NT	-
	(**10**)	151	-	-	-	-	-	-	-	-	+++	NT	-
Tala- Mokolo propolis /2010	PECE **6**	78.6	-	NT	++	-	+	Traces	+	+	++	NT	+
	(**13**)	130	-	NT	-	-	-	-	-	-	+++	NT	-
	(**14**)	280	-	NT	-	-	-	-	-	-	+++	NT	-

+ = positive; ++ = intense; +++ = very intense; NT = Not Tested;
PHE: *Propolis* Hexane Extract; PEAE:*Propolis* Ethyl Acetate Extract; PME:*Propolis* Methanol Extract; PMWE: *Propolis* Methanol-Water Extract; PMRE: *Propolis* Methanol Residue Extract; PECE: *Propolis* Ethanolic Crude Extract.

Table. 6: ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR data for compounds 7 and 8 in $CDCl_3$.

Position	7		8	
	δ_H(mult.,*J* in Hz)	δ_C	δ_H(mult.,*J* in Hz)	δ_C
1	α 1.47 (m); β 1.59 (m)	30.4	α 1.14 (m); β 1.22 (m)	30.4
2	α 1.42 (m); β 1.42 (m)	31.9	α 1.85 (m); β 1.93 (m)	29.9
3	α 3.12 (dd ; 6.4 and 9.2)	78.8	α 3.20 (dd; 6.4 and 9.2)	78.8
4	/	39.0	/	45.3
5	α 1.41 (dd; 9.0/9.0)	52.0	α 1.11 (dd; 12.0/2.2)	48.8
6	α 1.42 (m); β 1.51(m)	32.8	α 1.64 (m); β 1.68 (m)	18.3
7	α 0.90 (m); β 1.10 (m)	26.0	α 1.41 (m); β 1.46 (m)	30.0
8	/	40.5	/	47.1
9	/	20.0	/	32.8
10	/	25.4	/	26.0
11	α 1.98 (m); β 2.04 (m)	39.0	α 2.09 (m); β 2.13(m)	32.0
12	5.42 (dd ; 3.6/2.3)	125.6	5.52 (dd; 5.0/1.2)	125.6
13	/	139.3	/	129.6
14	/	45.3	/	48.0
15	α 1.75 (m); β 1.82 (m)	28.1	α 1.48 (m); β 1.52 (m)	36.4
16	α 0.92 (m); β 0.94 (m)	26.4	α 1.49 (m); β 1.71 (m)	28.1
17	/	48.8	α 2.32 (m)	52.0
18	β 1.35 (d)	47.9	0.71 (s)	14.0
19	α 1.15 (ddd; 10.5/9.5/6.5)	47.1	α 0.25 (d; 4.0); β 0.49 (d; 4.0)	26.4
20	β 1.31 (m)	36.4	α 0.17 (m)	35.6
21	α 1.15 (m); β 1.24 (m)	35.6	0.77 (d; 6.3)	19.3
22	α 0.91 (m); β 1.20 (m)	26.1	α 1.17 (m); β 1.29 (m)	39.0
23	1.05 (s)	26.0	α 1.24(m); β 1.25 (m)	25.4
24	0.98 (s)	18.1	α 1.49(m); β 1.56 (m)	40.5
25	α 0.18 (d; 5.8); β 0.39 (d; 5.8)	30.0	/	139.3
26	0.65 (s)	14.0	α 4.78(d; 1.6); β 4.79 (d; 1.6)	114.0
27	1.16 (s)	29.9	1.76 (s)	20.0
28	0.66 (s)	21.1	0.88 (s)	26.1
29	β 0.75 (d)	18.3	0.82 (s)	18.1
30	α 0.66 (d)	21.1	1.23 (s)	21.1

Assignments were established by DEPT 135, ^{1} H-^{1}H COSY, HSQC and HMBC data.

seven of them are attributed to methyl groups, one for oxymethine at δ_C 78.8 as well as a signal for methylene at δ_C 30.0, correspond to cycloartane type triterpene. This structure was confirmed by the unsaturation number (7) and ^{1}H-^{1}H COSY, HSQC and HMBC data. The upfield shift of the methyl group at δ_C 18.1 (CH_3-29) and ^{1}H-^{1}H COSY correlation between oxymethine proton at δ 3.12 showed the presence of OH group at C-3. All presented data led to the identification of **7** as 25-cyclopropyl-3β-hydroxyurs-12-ene.

Compound **8** showed a pseudo-molecular ion peak $[M+Na]^+$ at *m/z* 447.36 in its ESI-TOFMS and the presence of 30 carbons in the ^{13}C NMR spectrum (Table 6), suggesting a molecular formula $C_{30}H_{48}O$.The ^{1}H-NMR spectrum exhibited signals, characteristic for cycloartane-type triterpene: two methylene protons at δ 0.25 and 0.49 (both d, J = 4.0 Hz), six methyl groups at δ 0.71 (s), 0.77 (d, 6.3), 0.82 (s), 0.88 (s), 1.76 (s), one olefinic proton at δ 5.52 (dd, J = 5.0, 1.2 Hz), two broad singlets for protons from exo-methylene group at δ 4.78 and 4.78, and one oxymethine proton at δ 3.20 (dd, J = 6.4, 9.2 Hz). The presence of 30 carbons (^{13}C NMR), amongst which seven were attributed to methyl groups, one to oxymethine at δ_C 78.8 as well as a signal to the methylene at δ_C 26.4. Detailed analysis of spectral data led to identification of **8** as a cycloart-3β-hydroxy-12, 25(26)-diene.

The botanical origin of the Cameroonian propolis is yet unidentified but the terpenic profile leads to the suggestion that its plant source should be some conifer plants given the fact that Coniferae species have been cited as plant source for some propolis constituents (Popova *et al*., 2009). The identification of the source requires further studies, including direct chemical comparison of propolis and plant material as well as observation of honeybee behavior as an evidence for the plant origin of propolis (Popova *et al*., 2009).

Till now, cycloartane triterpenes have been isolated only from Brazilian and Cretan propolis (tropical type) and *Mangifera indica* (Anacardiaceae) have been shown as a plant source (Da Silva *et al*., 2005; Popova *et al*., 2009).

All the results obtained demonstrate that Cameroonian (Adamawa and Far Nord regions) propolis is a specific propolis type with triterpenes as major constituents. Further investigations on the chemistry of other samples from the Cameroonian region are needed in order to find out the area of distribution and the chemical variability of this brown propolis, as well as its plant source.

The isolated compounds and propolis crude extracts were tested on three environmental bacteria (Table 7) to screen their antimicrobial activity following disc diffusion method by measuring the diameters of zones of growth inhibition around the paper discs on agar plates. Minimum inhibitory concentration (MIC) values in mg/ml for the most active compounds and crude extracts were determined. The results showed interesting and promising antimicrobial activity (Table 8).

Results showed that all the propolis crude extracts failed to exhibit any antimicrobial activities against the test organisms except the methanol crude extract (**5)**, compounds **7, 8** and **10**. The methanol crude extract (**5)** had a broad spectrum activity by inhibiting both the growth of *E. coli* and *Ps. aeruginosa*. On the other hand, **7**, **8** and **10** showed significant inhibitory activities only against *Salmonella* spp. Their specific inhibitory actions against *Salmonella* may be due to the fact that they are all triterpenes though belonging to three different classes (ursane, oleanane and cycloartane respectively). This confirms the fact that terpenoids contribute to the biological properties of propolis besides phenolic compounds and flavonoids (Bankova *et al*., 1996; Matsuno, 1995; Pereira *et al*., 1999). Their antibacterial properties against *Salmonella spp* and *E.coli* may explain their traditional use (PROMAX-C) in the treatment of infectious diseases in Cameroon. This result is very close to the work of Yamauchi *et al* (1975) who reported that olean-12-en-3, 16, 23-triol activity was negative against *E. coli*. In fact, this product differs from **7**, **8** and **10** by having 3-OH groups instead of one in **7**, **8** and 2-OH in **10.** Compounds **8** and **7** had similar activities against *Salmonella*. This could be due to the fact that they are both cycloartane type triterpenes (Fig.1). Surprisingly, compounds **9, 13** and **14** did not shown any antimicrobial activity against all the bacteria tested and no clear reason can be given to this peculiar behaviour.

The MIC values recorded in Table 8were interestingly very low (0.1-0.2 mg/ml) indicating a very high antibacterial activities of compounds **5**, **7**, **8** and **10.** Their antibacterial activities were found to be superior to those of Amoxicillin (0.1-0.2 versus 0.4). Compounds **7, 8** and **10** had high MIC values against *Salmonella* (0.1–0.15 mg/ml), while MeOH crude extract (**5**) exhibited also moderate but broad spectrum activity against both *Ps. aeruginosa* and *E. coli* (MIC value 0.2 mg/ml). The specific inhibitory effects of compounds **7**, **8** and **10** against *Salmonella* might be due to the fact that they are pure compounds (triterpene as the major component) thereby lacking synergistic substances. These three triterpenes may be exploited for the treatment of salmonellosis if they exhibit antimicrobial effects against other strains of non-typhoid *Salmonella*. On the contrary, the broad spectrum activities of MeOH crude extract (**5)** against *Ps. aeruginosa* and *E. coli* could explain the presence of several synergistic bioactive compounds (alkaloids, reducing compounds, saponins, coumarins and tannins).

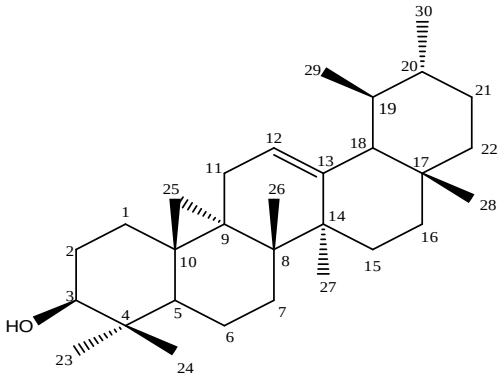
The strong inhibitory activities of the compounds **5**, **7**, **8**, **10** against environmental Gram negative bacteria may suggest that they could exhibit greater activities against Gram positive organisms given the fact propolis extracts in general have been reported to be more harmful to Gram positive organisms (Grange *et al*., 1990; Kalogeropoulos *et al*., 2009; Popova *et al*., 2009; Seidel *et al*., 2008). The results of the Cameroonian propolis on the tested organisms in this study are encouraging in comparison with the previous works (Mbawa *et al*., 2010; Seidel *et al*., 2008). It is interesting to note that compounds **7**, **8**, and **10** are more active than the original extract (**5**) but lose their broad spectrum potency in the course of purification. However, further pharmacological and toxicity studies currently going on in the laboratory are necessary to establish if they could be safely used as topical antimicrobial agents.

Table. 7: Antibacterial activities (diameter of zone of inhibition-in mm) of the stock solutions of the propolis extracts. .

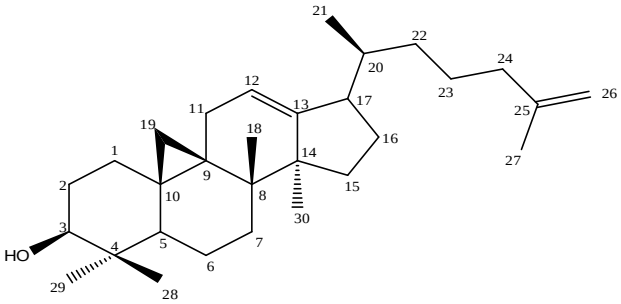
Tested extracts and isolated compounds	*E. coli*	*P. aeruginosa*	*SalmonellaSpp*
1: PHE (Ngaoundal)	-	-	-
2: PHE (Meiganga)	-	-	-
3: PEAE (Ngaoundal)	-	-	-
4: PEE (Tala-Mokolo)	-	-	-
5: PME (Meiganga)	26	32.5	-
6: PMRE (Ngaoundal)	-	-	-
7: 25-cyclopropyl-Urs-12-en-3 β-ol	-	-	75
8: Urs-12-en-3 β,-ol	-	-	46
9: Lup-20(29)-en-3-one	-	-	-
10: Olean-12-en-3 β, 28-diol	-	-	62.5
13: Lup-20(29)-en-3β-oate	-	-	-
14: 3β-hydroxylup-20(29)-ene	-	-	-
Reference: Control disc with (10% DMSO)	-	-	-
Amoxicillin	-	6	-

Table. 8: . Minimal inhibitory concentration (MIC) mg/ml of the propolis crude extracts and the isolated compounds showing antibacterial activities.

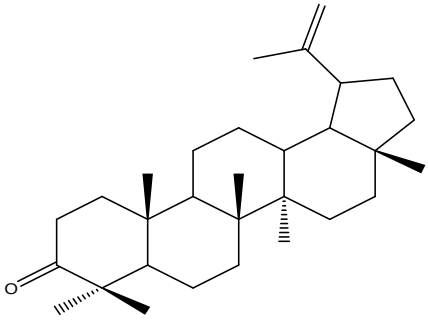
Tested extract and isolated compounds	*E. coli*	*Ps. aeruginosa*	*Salmonella***spp**
5: PME (Meiganga)	0.2	0.2	-
7: 25-Cyclopropyl-urs-12-en-3 β-ol	-	-	0.1- 0.15
8: Urs-12-en-3β,28-diol	-	-	0.15- 0.20
10: Olean-12-en-3 β,28-diol	-	-	0.1- 0.15
Amoxicillin	-	0.4	-



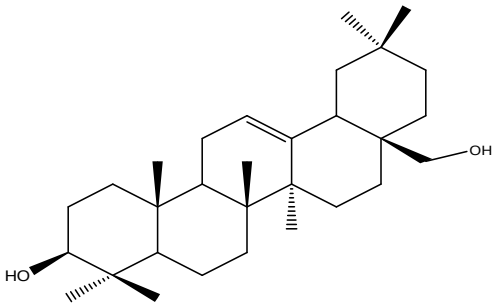
7 : 25-cyclopropyl-3β-hudroxyurs-12-ene



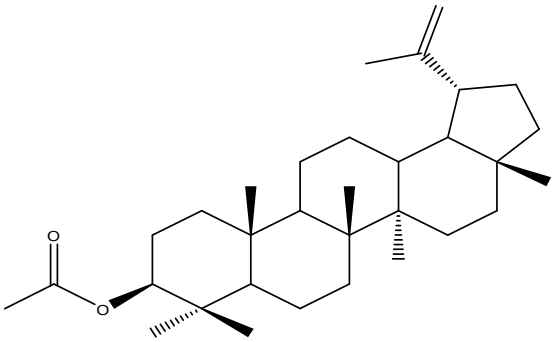
8 : cycloart-3β-hydroxy-12, 25(26)-diene



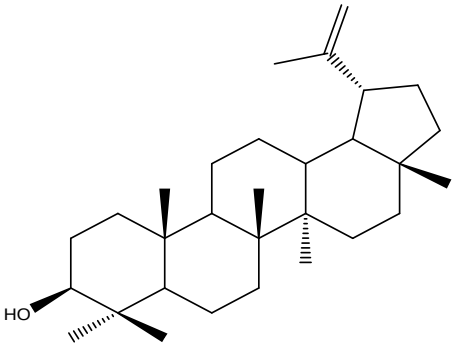
9 : lup-20(26)-en-3-one



10 : olean-12-en-3β, 28-diol



13 : lup-20(29)-en-3β-oate



14 : 3β-hydroxylup-20(29)-ene

Fig. 1: Structures of the isolated compounds.

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